REMARKS

Claims 1, 3-18, 21, and 22 are pending in this application. Claims 2, 19, and 20 have been cancelled. Claims 1 and 3-18 are currently amended. Claims 21 and 22 are new.

Principal attorneys for the Applicant have reviewed the pending Office Action and the cited references, and have the following remarks.

In connection with the objections regarding the novelty and the inventive step, the applicant respectfully is in disagreement with the Examiner's criterion.

In the proposed solution the applicant claim for a composition which allows the simultaneous identification of coliforms, differentiating them from other Gram-negative bacteria, and at the same time, Klebsiella and Citrobacter species are also differentiated from the rest of the coliforms. No of the previously cited media are able to do so. Our solution also offers the possibility to identify and count these microorganisms simultaneously. It should be note that the combination of the substances of protein origin that include, for example pancreatic or papaic beef heart hydrolysate and egg yolk proteins in the present invention, are used for the first time in this invention. Although the ratio of components in the composition of the cited patent is within the claimed ranges, the examiner should note that in both documents, the components of the substances of protein origin are different and the components of the present invention have a higher effect in comparison with other of state of the art.

In the composition of the present invention, at the same time we can also identify Salmonella, differentiating Salmonella typhi from non-typhi, and the first one from the rest of the microorganisms. In the Rambach Patent, only Salmonella non-typhi grows, and in the Mach Patent, grows, but could not be identified, neither differentiated.

Only in our composition Pseudomonas could be identified and growth properly. In any of the both cited patents in the state of the art, it could be done.

The media mentioned in the Atlas Book, are not intended for counting coliforms bacteria, being these media liquid.

As it is shown in the enclosed Table A, any of the existing composition cited by the examiner covers all the applications simultaneously as these applications are covered by the present invention.

The inclusion of substances of protein origin is original and was not previously describe in the state of the art. None of the cited Patents or the media in the Atlas Handbook contain the combination of beef heart pancreatic or papaic digest, together with dried egg yolk proteins and the rest of the hydrolysates.

It is not obvious that such combination yield in a composition able to grow a wide range of Gram-negative microorganisms and also able to allow identification reactions in the media for different species of microorganisms.

Beef heart hydrolysate was used in the formulation of the Columbia CNA Agar from Dalynn Biologicals (3 g/L) for the growth of Gram-positive microorganisms, and in this medium Gram negative microorganisms are suppressed. It means that this hydrolysate was used for a purpose complete opposite to that, we are claiming in our composition. It is not obvious that this hydrolysate promote the growth of Gram-negative microorganisms as we demonstrated in our composition.

In another composition, Heart Peptone was used to growth Listeria sp. (OXFORD Listeria Agar from Quelab) (3 g/L). As it is known, Listeria species have nothing in common (in the physiology and nutritional requirements sense) with Gram-negative bacteria. Further more, in this medium, Gram-negative bacteria are inhibited, for example E. coli should not grow at all, or grow very inhibited.

It is not obvious that heart peptone will support the abundant growth of Gram-negative microorganisms, in a composition where they should overcome the inhibitory effect of the added inhibitors.

The composition of heart peptone differs from the rest of the meat or bacteriological peptones, or peptones of animal tissue related in the Atlas handbook as component of culture media.

In the next Table B, we show the differences in the composition between average meat peptone or bacteriological peptone and beef heart pancreatic digest.

TABLE B

Compound	Meat peptone	Enzymatic digest of animal tissue	Bacteriological Peptone	Beef heart hydrolysate
Ca, ppm	200 - 300	500	344 - 690	20
Mg, ppm	288- 500	Not reported	206 - 355	600
Glutamine, %	7.90 - 11.50	9.67	9.93 - 12.50	12.90 - 18.40
Isoleucine, %	1.49 - 3.30	2.31	1.02 - 3.81	4.00 - 4.48
Leucine, %	3.31 - 6.00	5.50	3.65 - 4.60	5.80 - 7.89
Serine. %	1.24 - 3.03	2.98	1.76 - 4.10	4.36

Papaic digest also differs from the peptones described as bacteriological or meat or enzymatic digest.

As can be observed, beef heart hydrolysate contains significant less content of Calcium in the composition and higher content of Magnesium (we included this

hydrolysate because it possess high content of Mg, and being a bivalent ion, it allows the profuse growth of Pseudomonas in the medium and allows the appearance of a fluorescence of this microorganism in a maximum of 24 hours).

The beef heart hydrolysate has higher content of essential amino acids for the growth of Gram-negative bacteria, such as Glutamine, Isoleucine, Leucine and Serine.

In the previous state of the art, heart peptone was used as a supplementary nutrient being in the formulation in maximum quantities of 3 g/L.

In our solution, it is a main ingredient within the group of the protein compounds of animal origin is the pancreatic or papaic digest of beef heart, and it is used in quantities from 5 to 7 % among this group of substances, a higher amount than those used in previous media.

From the other point of view, egg yolk proteins ore used in culture media, but for a purpose completely different to the objective of its use in our composition.

In the Atlas Handbook, egg yolk proteins ore used in media, for the growth of Gram-positive bacteria, not for the growth of Gram negative. The aim of the inclusion of egg yolk in the formulation of Gram-positive media such as Lipovitelin Salt Manitol Agar is to detect the lipovitelin-lipase activity of Staphylococcus aureus and other bacteria.

Other media which include egg yolk emulsion with the purpose of detecting enzymatic activity are: Baird Porker Agar, Cereus Selective Agar, Perfringens Agar Base, KRANEP Agar, and Anaerobic Egg Agar.

No other previous solution includes the use of dehydrated egg yolk proteins, not in form of an emulsion, but in a powdered form, and not for detecting enzymatic activity, but for new not obvious applications: the promotion of on abundant growth of Gram negative microorganisms, and for to increase the contrast of the chromogenic reaction and of the alcohol splitting reaction.

On the other hand, in connection with the objection regarding to the claims 1, 2, 7, 8, 10-13 and 18 the examiner should note that in the Rambach US potent No. 5,194,374 there is no reference about the use of salts of magnesium, sodium carbonate and low molecular weight nitrogen compounds, not in the claims, neither in the examples.

The examiner put emphasis on the fact that the composition claimed in our solution has the some purpose as the Rambach one, but we can affirm that it is not intended for the some purpose, because in our case the target microorganisms are not only Salmonella, but also Citobacter, Klebsiella, other coliforms, and Pseudomonas at the some time. It means that we use a composition with a number of known substances (agar, peptones, pH indicators) and other not known for the intended purpose (hydrolysate of beef heart, powdered egg yolk protein) and new substance in culture

media (diatomaceous earth- 3MgOx4SiO2xH2O) to obtain a new, not previously described result: the simultaneous identification, differentiation and count of this wide range of Gramnegative microorganisms in one steep in one plate.

The examiner cited that some components of the Rambach medium contain several of these elements, for example, it is known that yeast extract has in its composition magnesium, sodium, and low molecular weight substances of protein origin, but they are in such few amounts that cannot be compared with the amount we are claiming for our composition.

In the next table C, we show the composition of various yeast extracts in relation with the following compounds

TABLE C

Components	Pasteur	BBL	BioMerieux	Merck	OXOID	MAST	Average
NaCl, %	< 10	2	0,5	-	0,3	0,4	2,64
Magnesium, %	0,25	0,065	0,07	0,05	0,000315	1,5	0,32
Creatine, (nitrogen compound of low molecular	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported	No

weight)							

The next calculation shows that in the Rambach formulation a very small amounts of these compounds are present, and these amounts are not enough for guarantee the growth of all the Gram-negative microorganisms that we are able to growth in our composition.

Taking only the example of the yeast extract content, we can see the differences in amounts:

Yeast extract in Rambach Agar - 4 % in the dry mixture of the medium, taking the average content of elements in yeast extract we get the following quantities of them in the final formulation:

Component	Rambach	Claimed composition
Na as NaCl, %	0,11	0,25 (yeast extract) + 0,50 = 0,75
Mg, %	0,01	0,03 (yeast extract) + 13,81 = 13,84%
Creatine, % (nitrogen compound of low molecular weight)	0	1,58

We include in our formulation the addition of such substances in order to guarantee an abundant growth of Grom-negotive microorganisms, as Salmonella, E. coli, Klebsiella, other coliforms, and Pseudomonas, overcoming the inhibitory effect of the inhibitors added to the composition, and also allowing the detection of the fluorescence and growth of Pseudomonas in the medium in a maximum of 24 hours.

In our experiments, we demonstrated that the use of yeast extract alone will not support or guarantee the growth and detection of Pseudomonas, and also the growth of all the target microorganisms no detected in Rambach patent.

See example No 1, Table 3 of our application, in the Rambach Agar variant, Enterobacter aerogenes did not grew at all, while in our composition, we obtained a growth of 60 CFU/mL, for E. coli. For Citrobacter in our composition the number of CFU was higher than in the Rambach one.

In the same example, but in Table 2, was demonstrated that Salmonella typhi and Proteus vulgaris were not able to growth in Rambach medium, mean while in our composition both microorganisms were adequately grown.

We consider that the claim No. 18 is essential for the present invention, because at this proper pH range and not at other range, the composition allows not only the growth of Salmonella, but also the growth of other Gram-negative organisms. Besides, at this pH range, we can guarantee the fluorogenic effect for detecting Pseudomonas,

and the chromogenic reactions for identification and differentiation of Gram-negative bacteria. There is not a single identification reaction, but several, for example the splitting of chromogenic compounds, and propanediol, the diffusion of reagents and of the products of the splitting reaction, among others, and every reaction has an optimal range of pH, not obvious in every case.

In our experiments, we suddenly observed new unexpected findings, for example, the growth with different color of Klebsiella.

It is not obvious that all the effects we can achieve in our composition we reach because we established a pH range characteristic for the growth of Salmonella. Even other media for Salmonella have a different pH range, for example Rappaport Vasiliadis medium has a pH of 5,5, lower than our, and Tetrationate Broth has a pH from 8 to 7,5-8,0.

We consider to keep the claim 7, because the use of bile salts or mixture of cholic and deoxycholic acids are essential for the present invention. These and not other inhibitors of the Gram-positve bacteria in the specified range of concentration allowed the growth of all Gram-negative bacteria and completely suppressed the growth of Gram-positve ones. In Rambach Agar, as we previously demonstrated, Enterobacter and Proteus were inhibited, while in our composition they profusely grew.

Other inhibitors of Gram-positve bacteria could not be used, because they interact with other components of our composition (with magnesium salts) or interfere with the chromogenic or fluorogenic reactions, for example brilliant green or crystal violet.

According to the examiner's observations that in the Atlas reference and in previous patents, the components of the claimed composition were previously described for the same purpose, once again we stated that the purpose of our composition differs from the purpose of all the previously described media.

In some cases we included components used previously in other media but for different purposes, in other cases we used new components, and at least we included components previously described for the same purpose, but we obtained a completely different results not achieved before with no of the media in which they were included.

We illustrate these statements with the following table D:

TABLE D

Components	**In the present invention**	**Media in the state of the art in which the components are included**	**Purpose of the previously described media**	**Advantages and new findings in the proposed invention**
Cystine and Cysteine	Promote the faster and more intense detection of propanediol splitting reaction by Salmonella	Selenite-Cystine Medium	Enrichment of Salmonella and inhibition of other bacteria including some Gram-negative, such as coliform bacteria in liquid medium	No inhibition of other Gram-negative bacteria. Faster identification of Salmonella. Allows the growth of Salmonella non-typhi. Allows the growth of Salmonella before 24 hours and without pre-enrichment. Allows the recovery of Gram-negative microorganisms directly from samples. Allows the count of each species of microorganisms
		Lysine Iron Agar	Medium for the detection of some reactions which together with other media allows the identification of Salmonella and other Gram-negative bacteria, but do not allow their recovery directly from samples or their quantitative count.	
		CLED medium Columbia Agar with Cysteine	General purpose media for urine samples	
		Corynebacterium medium	Support the growth of Corynrbacterium species (No relation with the microorganisms identified in present invention)	

		Medium with Ribise and Cystine	Support the growth of Plevotus ostreatus (No relation with the microorganisms identified in present invention)	
		Cell culture media	Inhibition of enzymatic activity in the proteosome (No relation with the microorganisms identified in present invention)	
Egg Yolk proteins	In a dried powdered form, for supporting the growth of Gram-negative microorganisms, having a high concentrated protein content. Allows the identification of colored reactions, serving as a contrast agent	Media for Staphylococcus aureus	Detection of Staphylococcus aureus by its lecitinase activity	Is used in our composition for growing Gram-negative microorganisms, with a completely opposite approach than the previous one: the growth of the Gram-positive microorganisms. The use as a contrast agent previously was not reported. The use as a dry, powdered egg yolk ingredient with higher protein content was described in the state of the art.
		Composition for the detection of enzymatic activity of Vibrio vulnificus	Detection of enzymatic activity of Vibrio vulnificus (No relation with the microorganisms identified in present invention)	

Magnesium chloride	For the achievement of the intensification and early appearance of the fluorescence reaction of Pseudomonas aeruginosa and to promote an abundant and fast growth of Gram-negative bacteria, specially Salmonella	Rappaport Vasiliadis Medium	Salmonella enrichment in the presence of other microorganisms. E. coli and coliforms are depressed	Simultaneous identification of Pseudomonas aeruginosa by the appearance of fluorescence in a maximum of 24 hours and abundant growth of Salmonellas, and coliforms and other Gram-negative bacteria
		Cetrimide Agar	Identification of Pseudomonas aeruginosa by pigmentation. Salmonella and other Gram negative bacteria including coliforms are inhibited or poor grow	
Creatine	For the achievement of the intensification and early appearance of the fluorescence reaction of Pseudomonas aeruginosa and to promote an abundant and fast growth of Gram negative bacteria.	No	No	
Diatomaceous earth (3MgOx4SiO2xH2O)	Promoting the fluorescence of Pseudomonas aeruginosa and contrasting agent	No	No	

In relation with the cited by the examiner work on iontophoresis (C.P. Davis, 1993) . This technique has no relation with the present invention. In this paper the author use creatinine for diminishing the damage of the Gram-negative microorganisms, but do not mention creatine (the compound we use in our composition). Creatine and creatinine have different composition and structure and a different role in metabolic pathway.

These facts, together with those exposed in the application document, show the novelty and superiority of our solution in relation with the previous state of the art, and can be resumed as a new composition that allows a simultaneous identification, differentiation and count of different species and subspecies of microorganisms not previously achieved.

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TABLE A

Microorganisms		Composition according to the present Invention	Rambach, US Patent 5,194,374	Mach, US Patent 5,723,308	Lysine Iron Cystine Neutral Red Broth (Atlas, p. 507)	Rappaport Vasiliadis Broth and modifications (Atlas, p. 763)	Selenite Cystine Broth (Atlas, p799)
Coliforms	E. coli	Good growth, blue-greenish colonies	Good growth, blue-green colonies	Good growth, yellow colonies, gas formation	**Not reported**	**Not reported**	**Not reported**
	Enterobacter sp.	Good growth, blue-greenish colonies	**Not claimed by the author, but in our experiments - inhibited in this medium**	Good growth, yellow colonies, gas formation	**Not reported**	**Not reported**	**Not reported**
	Klebsiella sp.	Good growth, violet colonies	**Not claimed by the author, but in our experiments - inhibited in this medium**	Good growth, yellow colonies, gas formation, **but could not be differentiated from other coliforms**	**Not reported**	**Not reported**	**Not reported**

Microorganisms		Composition according to the present invention	Rambach, US Patent 5,194,374	Mach, US Patent 5,723,308	Lysine Iron Cystine Neutral Red Broth (Atlas, p. 507)	Rappaport Vasiliadis Broth and modifications (Atlas, p. 763)	Selenite Cystine Broth (Atlas, p799)
	Citrobacter sp.	Good growth, violet colonies	Good growth, blue violet colonies	Good growth, yellow colonies, gas formation, **but could not be differentiated from other coliforms**	**Not reported**	**Not reported**	**Not reported**
	Others	Good growth, blue-greenish colonies	**Not claimed by the author**	Good growth, yellow colonies, gas formation	**Not reported**	**Not reported**	**Not reported**
Salmonella	Salmonella non typhi	Good growth, red center, **clearer borders colonies**	Good growth, red colonies	Good growth, **but could not be identified, nor differentiated**	Good growth, red color medium, **but could not be differentiated from other Salmonella**	Good growth, **but could not be identified, nor differentiated**	Good growth, **but could not be identified, nor differentiated**
	Salmonella typhi	Good growth, transparent colonies	**Not claimed by the author, but in our experiments - inhibited in this medium**	Good growth, **but could not be identified, nor differentiated**	Good growth, red color medium, **but could not be differentiated from other Salmonella**	Good growth, **but could not be identified, nor differentiated**	Good growth, **but could not be identified, nor differentiated**

Microorganisms		Composition according to the present invention	Rambach, US Patent 5,194,374	Mach, US Patent 5,723,308	Lysine Iron Cystine Neutral Red Broth (Atlas, p. 507)	Rappaport Vasiliadis Broth and modifications (Atlas, p. 763)	Selenite Cystine Broth (Atlas, p799)
Pseudomonas aeruginosa		Good growth, orange color with darker center colonies, green yellow fluorescence	**Not claimed by the author**	**Not claimed by the author**	**Not reported**	**Not reported**	**Not reported**
Proteus sp.		Good growth, transparent colonies	Good growth, colorless colonies	(Proteus vulgaris) - Yellow colonies without gas	**Not reported**	**Not reported**	**Not reported**
Other Gram negative bacteria		Good growth, colorless colonies	**Not claimed by the author**	Good growth, without change	**Not reported**	**Not reported**	**Not reported**

Bold letters- marked differences between the new proposed composition and the existing solutions

"Not claimed by the author" or "Not reported" - means that the mentioned media were not designed to growth the indicated microorganisms and these microorganisms do not grow at all, or do not grow properly (inhibited) or in few cases, if grow, could not be differentiated or identified.